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(54) Title: STABILIZED AQUEOUS LIQUID FORMULATIONS OF PHYTASE

(57) Abstract

The present invention provides stabilized aqueous liquid formulations having phytase activity which exhibit increased resistance to heat inactivation of the enzyme activity and which retain their phytase activity during prolonged periods of storage. The liquid formulations are stabilized by means of the addition of urea and/or a polyol such as sorbitol and glycerol as stabilizing agent. Also provided are feed preparations for monogastric animals and methods for the production thereof.

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STABILIZED AQUEOUS LIQUID FORMULATIONS OF PHYTASE

The present invention relates to liquid formulations of enzyme activities and in particular, aqueous liquid formulations containing phytase activity.

Background of the Invention

Because of their ease in handling, liquid formulations of enzymes are in many cases easier to use in industrial applications, such as in the preparation of animal feeds. However, it is often true that enzymes in liquid formulations are not stable and suffer from inactivation during prolonged storage or by exposure to high temperature. This is especially true of non-purified solutions of enzymes which are directly obtained from a fermentation broth after subsequent filtration and ultra-filtration. Ultra-filtered fermentation broths are often preferred to purified enzyme solutions since purification often leads to a significant decrease in overall enzyme activity yield and furthermore, a great deal of the production costs arise from purification processes. For many industrial applications, a purified enzyme preparation is simply not necessary.

Ultra-filtrates of fermentation broths containing the enzyme phytase may, for example, be directly applied to the diets of monogastric animals in order to release inorganic phosphorous from the anti-nutritional factor phytate, thus avoiding the necessity of adding phosphorous to the feed and consequently lowering the amount of phosphorous found in the excreta of the ingesting animal, an obvious benefit for the environment (see European Patent Application 420,358 and U.S. Patent 3,297,548).

Since phytase is applied to the animal diets in lieu of added phosphorous, it is necessary to ensure that the enzyme is homogenously applied to the feed in order to provide the animal ingesting the feed with its required amount of dietary phosphorous. A liquid formulation of the phytase enzyme would

thus be advantageous since it may be evenly applied via a means such as spraying, thus providing a homogenous feed product.

U.S. Patent 3,297,548 does in fact mention liquid preparations of phytase, wherein said liquid form of the phytase enzyme is prepared from a concentrate of the enzyme obtained by heating a filtered fermentation broth at 55-70°C under vacuum.

However, it has been observed that the exposure of aqueous phytase-containing fermentation broth ultra-filtrates to temperatures in excess of 45°C leads to the rapid inactivation of the enzyme activity (see Figure 1).

It would thus be beneficial to obtain a stabilized aqueous liquid formulation containing phytase activity which could be easily applied, inter alia, to the feeds of non-ruminant animals.

One method of obtaining a stabilized enzyme preparation is the addition of stabilizing agents. Examples of various stabilizing agents which have been applied to enzyme formulations are polyols (e.g. glycerol, sorbitol, sucrose, glucose, lactose), ions (e.g. salts, osmolytes, metal ions such as calcium), ethylene glycol, dialkylsulphoxides, dioxin, polymers (e.g. polyethylene glycol, hydroxyethylcellulose), primary alcohols and substrates and similar ligands. Application studies of various enzyme stabilizing agents are summarized in the review articles of Klibanov, A.M. (Advances in Applied Microbiology, vol. 29 (1983: Academic Press; Laskin, A.I., ed.), pp. 1-28), Carpenter, J.F. et al. ((1990) J. Dairy Sci., vol. 73, pp. 3627-3636) and Gray, C.J. ((1988) Biocatalysts, vol. 1, pp. 187-196).

Summary of the Invention

The present invention provides stabilized aqueous liquid formulations having between 100-20,000 units of phytase activity per gram formulation, which exhibit increased resistance to heat inactivation of the enzyme activity and

which retain their phytase activity during prolonged periods of storage.

According to the present invention, it has been found that the phytase enzyme may be stably maintained in a liquid formulation containing stabilizing agents such as polyols or in a liquid formulation containing low concentrations of urea. The stabilized liquid formulations of the present invention are more resistant to heat inactivation of enzyme activity and may be stored for longer periods of time with better retention of phytase activity. Mixtures of polyols and urea as stabilizing agents are also encompassed by the liquid formulations of the present invention.

The liquid formulations of phytase are applicable to a number of industrial applications requiring phytase activity. In particular, the liquid formulations of phytase may advantageously be applied to the feeds of monogastric animals as an alternative to the addition of phosphorous. Means such as spraying may be used to apply the enzyme evenly to the feed to ensure the homogeneity of the liberation of phosphorous from phytate throughout the feed product.

Brief Description of the Figures

Figure 1 Thermostability of a phytase-containing ultra-filtrate (without added stabilizing agents).

Figure 2 Stability of a phytase-containing ultra-filtrate (containing 0, 2, 5, 10 and 25% urea and 25% sorbitol, respectively) at 35°C.

Figure 3 Stability of a phytase-containing ultra-filtrate (containing 0, 25 and 50% sorbitol and 0, 25 and 50% glycerol, respectively) at 30°C.

Detailed Description of the Invention

The present invention provides stabilized aqueous liquid formulations of the enzyme phytase characterized in that the liquid formulations contain stabilizing agents such as polyols, low concentrations of urea or mixtures thereof. The stabilized liquid formulations of the present invention are

further characterized by their improved resistance to heat inactivation of enzyme activity and furthermore may be stored for longer periods of time with better retention of phytase activity.

It is especially remarkable that urea should have a stabilizing effect on the liquid formulation of phytase since until now it has generally been accepted that urea destabilizes proteins in solution and enhances the inactivation of enzymes (see Carpenter, J.F. et al., supra).

Phytase activity is preferably obtained from a microbial source such as bacteria, fungi and yeasts. Particularly preferred phytases are those having good stability in acid environment such as those obtainable from fungi. Especially preferred are phytases obtainable from species of the fungal genus Aspergillus, particularly from the species Aspergillus ficuum, Aspergillus niger, Aspergillus awamori, Aspergillus oryzae and Aspergillus nidulans, and most preferably from the species Aspergillus ficuum and Aspergillus niger.

The desired phytase activity may be produced by fermentative means, such as that described in U.S. Patent 3,297,548. Alternatively, phytase is preferably produced in larger quantities using recombinant DNA techniques such as described in European Patent Application 420,358. In a preferred embodiment, a fungus of the species Aspergillus (especially Aspergillus niger), which has been transformed with the phytase-encoding gene obtained from the species Aspergillus ficuum, is cultured under conditions conducive to the expression of the phytase-encoding gene, as described in European Patent Application 420,358.

The phytase-containing fermentation broth is preferably treated by means of both filtration and ultra-filtration prior to being used in the liquid formulation of the present invention.

In a preferred embodiment of the present invention, following fermentation, the phytase-containing medium is filtered using a 0.2 μm filter to remove cells and other solid debris from the solution followed by sterile

filtration. This filtrate is then subjected to ultra-filtration using a 5-10 Kda filter. The thus-obtained ultra-filtrate may then be used directly in the liquid formulations of the present invention.

The pH of the phytase-containing ultra filtrate will preferably be in the range of pH 2 to pH 6. More alkaline pH values have been observed to lead to gel formation. Optimally, the pH will be in the range of pH 3 to pH 5.

The amount of phytase activity in the stabilized liquid formulation may be between 100-20,000 Units Phytase Activity per gram total formulation. Preferably, the liquid formulation will contain between 1,000-10,000 Units phytase activity per gram total formulation. Most preferably, the liquid formulation will contain about 5,000 Units phytase activity per gram total formulation.

Phytase activity of a sample containing urea was measured as follows: a sample containing phytase is diluted until it contains an estimated 0.02-0.08 units of phytase activity per ml. This sample is incubated with 5 mM sodium phytate in 0.25 M sodium acetate buffer, pH 5.5 at 37°C. The reaction is terminated after 60 minutes by the addition of a molybdate-vanadate reagent solution (composition: 250 ml of a 100 g/l ammonium molybdate solution; 250 ml of a 2.35 g/l ammonium vanadate solution; 165 ml 65% nitric acid; diluted with water to 1 l total volume) and the amount of phosphorous released is determined by measuring the yellow color of the vanadomolybdophosphor complex spectrophotographically (415 nm) and comparing to a standard dilution curve.

Phytase activity of a sample containing a polyol was measured as follows: a sample containing phytase is diluted until it contains an estimated 0.02-0.08 units of phytase activity per ml. This sample is incubated with 7 mM sodium phytate in 0.25 M sodium acetate buffer, pH 5.5 at 37°C in a total volume of 5 ml. The reaction is terminated by the addition of 2.5 ml of a solution of trichloroacetic acid (120 g/l) and iron(III)chloride (4.3 g/l). The precipitate is removed by centrifugation for 10 minutes and 3000 x g. To 1

ml of the supernatent, 1 ml ascorbic acid solution (10 g/l) and 8 ml molybdate solution (0.6 g/l ammonium molybdate tetrahydrate, 0.03 g/l potassium antimonoxotartrate and 0.6% sulphuric acid) are added. The resulting blue color is measured spectrophotometrically at 720 nm. The results are compared with a standard dilution curve prepared with a phosphate standard solution.

A unit of phytase activity is defined as the amount of enzyme which is able to release 1 μmol phosphate per minute in either of the assays described above.

As mentioned above, the use of stabilizing agents such as water-soluble polyols and/or urea form an integral part of the present invention. Of the stabilizing agents, urea is most preferred since it is generally used in lower concentration and provides a less viscous solution which may be easier to apply.

Preferred water-soluble polyols for use in the present invention are sorbitol, glycerol, polyethylene glycol (especially PEG 6000) and propylene glycol. Especially preferred polyols are sorbitol and glycerol. The water-soluble will be present in an amount of at least 5% (w/w). It will be understood by those skilled in the art that the upper limit of water-soluble polyol present in the formulation will depend on the application thereof and that high concentrations of water-soluble polyols (above 60% (w/w)) are often too viscous to be easily used. The amount of water-soluble polyol present in preferred liquid formulations of the present invention is between 25-60% (w/w), more preferably between from about 25 to about 50% (w/w) and most preferably between from about 35 to about 50% (w/w).

Urea may normally be applied to the liquid formulation of the present invention in amounts ranging from greater than 1% (w/w) to 10 % (w/w). Preferably, urea will be present in the liquid formulation in the range of between 2-10% (w/w) and most preferably about 5% (w/w).

For optimal longevity, the phytase-containing liquid formulation will preferably be stored at temperatures not

exceeding 35°C and more preferably not exceeding 30°C and most preferably not exceeding 25°C.

The phytase-containing liquid formulations of the present invention display an increased stability and retention of phytase activity upon extended storage periods as well as increased resistance to heat inactivation (see Figures 2 and 3; compare Figure 1).

The liquid formulations of the present invention may be applied to a variety of industrial applications requiring phytase activity such as in animal feeds, soy processing and wet milling of grains.

In a preferred embodiment, the liquid formulations of the present invention are applied to feed compositions for monogastric animals, thus achieving the breakdown of the anti-nutritional factor phytate and the liberation of inorganic phosphorus for use by the animal ingesting the feed.

The phytase-containing liquid formulation may be applied directly to the feed, or alternatively be diluted prior to use to provide the desired amount of units of phytase activity per kg feed. The amount of phytase activity normally added to the feed is sufficient to provide a feed composition containing at least 50 Phytase Units per kg feed. Preferably, between 100-600 Phytase Units are added per kg feed. The amount of Phytase Units added to the feed will depend on the composition of the feed itself. Feedstuffs containing lower amounts of available phosphorous will generally require higher amounts of phytase activity and may easily be determined by the skilled artisan.

In a preferred embodiment, the phytase-containing liquid formulation is added to the feed by means of spraying after pelleting and/or extrusion of the feed, thus avoiding the high temperatures (50-120°C) regularly reached during the processing and pelleting of feed compositions. Moreover, spraying allows for even application of the enzyme, ensuring that the product will be homogenous in its content of phosphorous which has been liberated by the action of phytase

on the phytate present in the feed. This avoids problems of phosphorous deficiency in monogastric animals which can result from the ingestion of feed wherein the phosphorous remains bound as phytate and thus is unavailable to the animal.

Feed compositions, after treatment with the liquid formulation of the present invention, may either be used directly or may be packaged and stored for distribution and later use.

The stabilized liquid formulations of phytase of the present invention may also be applied to other industrial processes requiring phytase activity such as in soy processing and in the production of inositol and inositol phosphates.

The following examples are provided so as to give those of ordinary skill in the art a complete disclosure and description of how to make and use the invention and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperature, pH, etc.) but some experimental errors and deviation should be accounted for. Unless indicated otherwise, temperature is in degrees Centigrade and pressure is at or near atmospheric.

Example 1

Stabilization of a liquid formulation containing phytase by addition of urea

A fermentation broth obtained from Aspergillus niger strain GAM4, transformed with the phytase gene obtained from Aspergillus ficuum according to the process as described in European Patent Application 420,358, was processed by filtration using a polypropylene filter (type 25300 AN; FYLTIS-MOTTE, France), followed by sterile filtration using a BECO Type KD3 filter (E. Begerow GmBH, Germany) and a Schenk AFF100 Z filter (Filterbau GmBH, Germany) and ultra-filtration using a Millipore Pelicon system filter (10 kDa

membrane (PTGC 0005)) to yield a brown-colored liquid containing 10,000 units of phytase activity per ml liquid. The pH of the liquid was approximately 4.0, and the dry matter content between 20-25%. The liquid was stored at various temperatures, and the activity level was monitored for a period of 8 weeks.

As is shown in Figure 1, a clear temperature-dependent decrease in phytase activity is observed. Based on these observations, storage at cool temperatures is required.

Various concentrations of urea (between 2 and 25% (w/w)) were added to the aforementioned ultra-filtrate, and the storage stability was investigated in the same manner.

As is shown in Figure 2, the phytase activity retained after 8 weeks at 35°C is increased from 30% in the control sample (no urea added), to 50% in the case of 2% added urea, and to 58% in the case of 5 and 10% added urea. The addition of 25% urea resulted in a dramatic loss of activity in the same storage trial.

Example 2

Stabilization of a liquid formulation containing phytase by addition of glycerol and sorbitol

A fermentation broth obtained from Aspergillus niger strain GAM4 (CBS 513.88), transformed with the phytase gene obtained from Aspergillus ficuum according to the process as described in European Patent Application 420,358, was processed by filtration using a polypropylene filter (type 25300 AN; FYLTIS-MOTTE, France), followed by sterile filtration using a BECO Type KD3 filter (E. Begerow GmBH, Germany) and a Schenk AFF100 Z filter (Filterbau GmBH, Germany) and ultra-filtration using a Millipore Pelicon system filter (10 kDa membrane (PTGC 0005)) to yield a brown-colored liquid containing 10,000 units of phytase activity per ml liquid. The pH of the liquid was approximately 4.0, and the dry matter content between 20-25%. The liquid was stored at 30°C, and the activity level was monitored for a period of 8 weeks.

As is shown in Figure 3, a clear decrease in phytase activity is observed in the control sample (no polyols added).

Glycerol and sorbitol were added in the concentration of 25 and 50% (w/w) to the aforementioned ultra-filtrate and the storage stability was investigated as described in Example 1.

As is shown in Figure 3, the phytase activity retained after 8 weeks at 30°C is increased from 56% in the control sample without addition of polyols, to 74% and 87% in the case of 25 and 50% (w/w) glycerol addition, respectively. Addition of 25 and 50% (w/w) sorbitol increased the retention of phytase activity after 8 weeks to 78 and 94%, respectively.

Example 3

Application of a stabilized liquid phytase formulation on feed pellets

A liquid phytase formulation containing 50% (w/w) sorbitol or glycerol was diluted 40-fold with tap water to yield a solution containing 125 phytase units per gram. A batch of piglet feed pellets (3 mm diameter; manufactured by UTD, Maarsen, the Netherlands) was prepared by preconditioning the feed meal at 90°C, pelleting and subsequent cooling to ambient temperature. The feed pellets were then transferred into a mechanical mixer supplied with a single pressure nozzle. The diluted formulation (0.4% by weight) was sprayed onto the feed pellets while being agitated to yield a homogeneous product with an added phytase activity of 500 units/kg feed pellets.

A reference sample was prepared by mixing Natuphos® (a solid phytase preparation manufactured by Gist-brocades N.V.; Delft, the Netherlands) 500 units/kg feed through the piglet feed meal, and pelleting as described above.

It was observed that during preconditioning and pelleting at this temperature approximately 90% of the activity of the dry Natuphos® product was lost. In contrast,

no activity losses occurred when the liquid phytase formulations were applied directly on the feed pellets.

In addition, it was observed that when the above liquid phytase formulations were applied to feed pellets, no activity loss occurred during a two week storage period at room temperature.

Example 4

Application of a stabilized liquid formulation of phytase containing sorbitol, before the pelleting stage

A liquid phytase formulation containing 50% (w/w) sorbitol is diluted 40-fold with tap water to yield a solution containing 125 phytase units per gram. A piglet compound feed meal (unpelleted form of piglet feed manufactured by UTD; Maarsen, the Netherlands) is mixed with the diluted formulation (0.4% by weight) described in Example 3. This mixture is subsequently preconditioned at 90°C and pelleted. A reference sample is prepared by mixing Natuphos® 500 units/kg feed through the piglet feed meal, and pelleting as described above.

It is observed that during preconditioning and pelleting at this temperature approximately 90% of the activity of the dry Natuphos® product is lost. In contrast, of the diluted sorbitol formulation, only approximately 50% of the added activity is lost during the process.

Claims

1. A stabilized enzyme-containing liquid formulation characterized in that the liquid formulation contains between 100-20,000 Phytase Units per gram total formulation, preferably between 1,000-10,000 Phytase Units per gram total formulation, and most preferably about 5,000 Phytase Units per gram total formulation; and a stabilizing agent.

2. The liquid formulation of claim 1, further characterized in that the stabilizing agent comprises from greater than 1% (w/w) urea to 10% (w/w) urea and preferably between 2 and 10% (w/w) urea and most preferably about 5% (w/w) urea.

3. The liquid formulation of claim 1, further characterized in that the stabilizing agent comprises a water-soluble polyol.

4. The liquid formulation of claim 3, wherein the water-soluble polyol is present in the amount of at least 5% (w/w), preferably between 25-50% (w/w) and more preferably between about 35-50% (w/w).

5. The liquid formulation of claim 3 or 4, wherein the water-soluble polyol is selected from the group consisting of sorbitol, glycerol, polyethylene glycol and propylene glycol and preferably selected from the group consisting of sorbitol and glycerol.

6. A method of preparing a feed composition for monogastric animals, characterized in that the feed is treated with a phytase-containing liquid formulation according to any one of claims 1-5.

7. The method of claim 6, wherein the feed is treated with at least 50 Phytase Units per kg feed and preferably between 100-600 Phytase Units per kg feed.

8. The method of claim 6, further characterized in that the phytase-containing liquid formulation is applied after either pelleting or extrusion of the feed.

9. A feed composition for monogastric animals characterized in that the feed has been treated with a liquid formulation according to any one of claims 1-5.

10. The feed composition of claim 9, further characterized in that phytase activity is present as at least 50 Phytase Units per kg feed, and preferably at least 100 Phytase Units per kg feed.

11. A method of providing a monogastric animal with its dietary requirement of phosphorous, the method characterized in that a phytate-containing feed composition is treated with an amount of a phytase-containing liquid formulation as defined in any one of claims 1-5 which is sufficient to liberate phosphorous from the phytate contained in the feed composition, the method being further characterized in that no additional phosphorous is added to the feed.

12. The method of claim 12 further characterized in that the phytase-containing liquid formulation is applied to the feed composition by means of spraying.

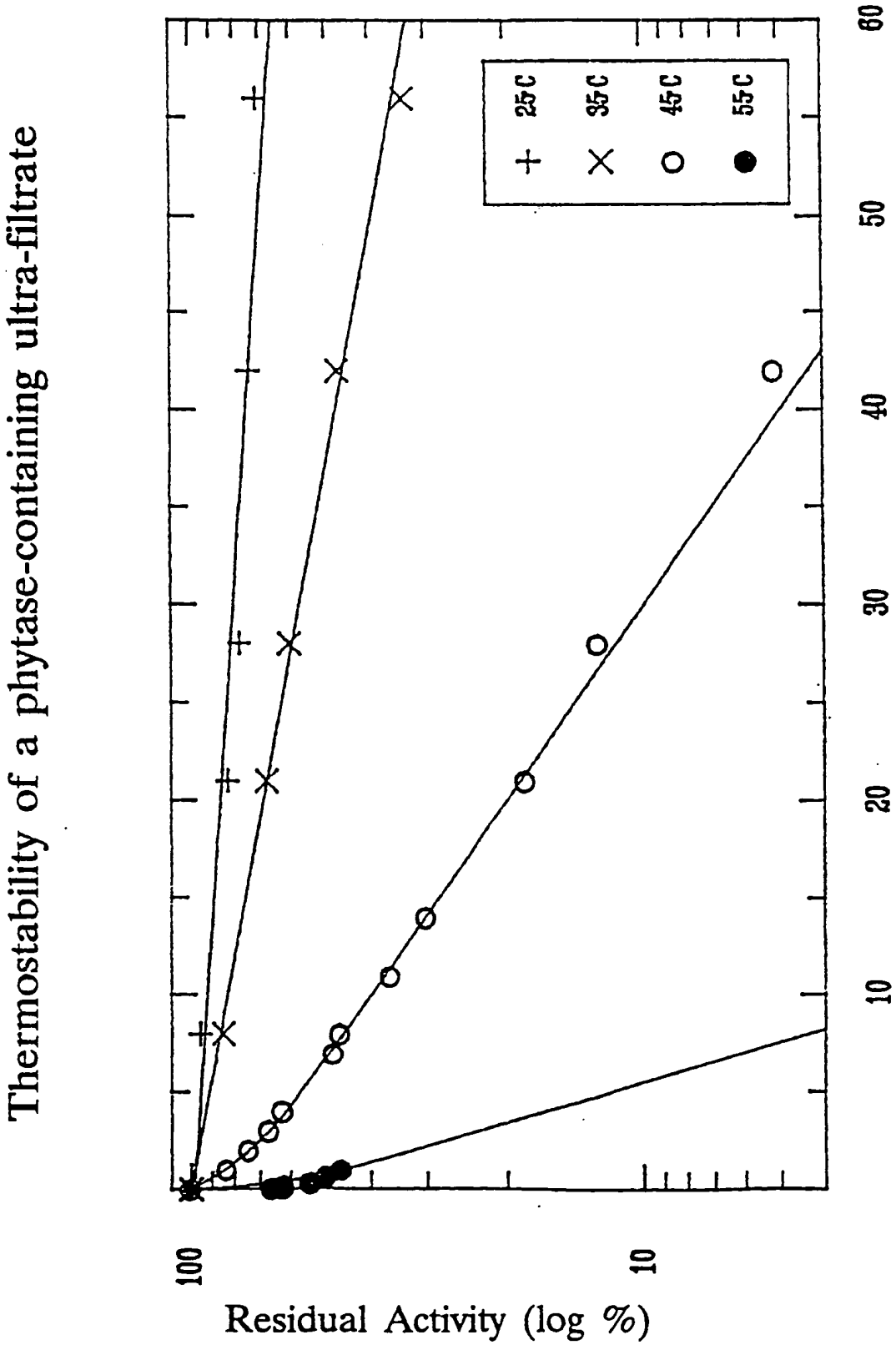
Thermostability of a phytase-containing ultra-filtrate
25°C
35°C
45°C
55°C
100
10
Residual Activity (log %)
10
20
30
40
50
60
Time (days)

Figure 1

Stability of a phytase-containing UF-concentrate with urea at 35 C

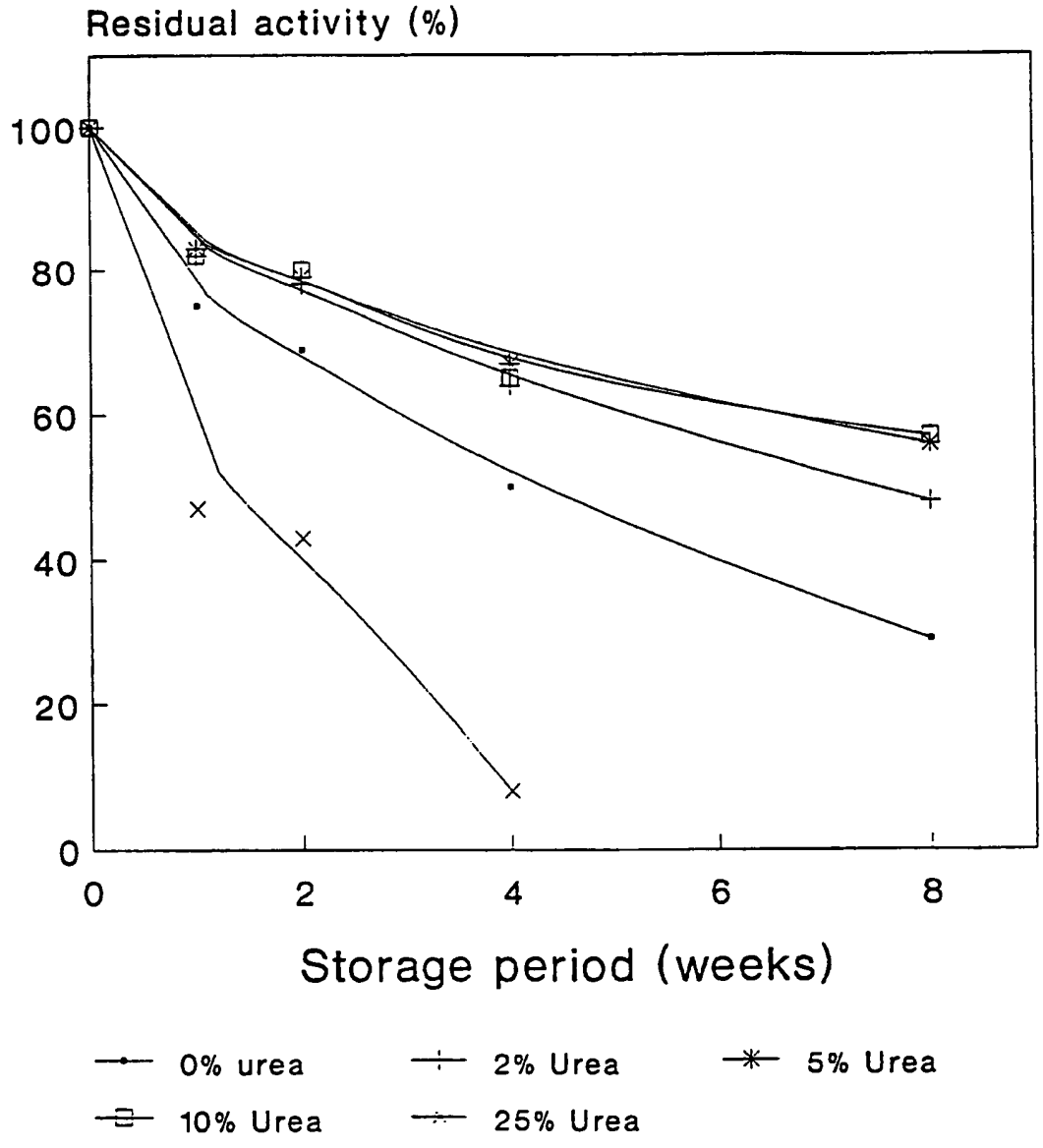

Figure 2

Stability of a phytase containing UF-concentrate with polyols

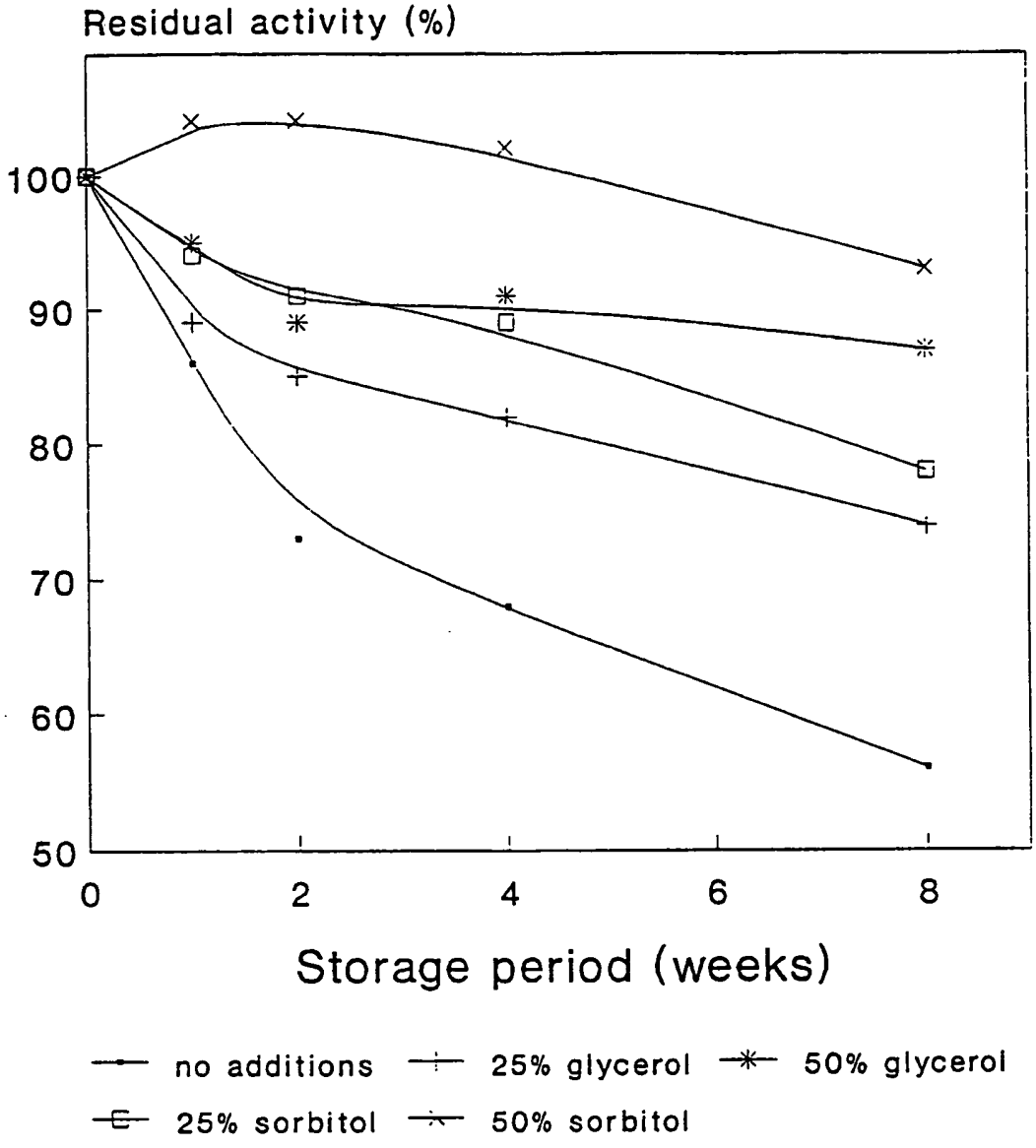

Figure 3

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

RECD 17 MAR 1993
WIPO PCT

INTERNATIONAL FORM

TO

Gist-brocades N.V.
Wateringseweg 1
Postbus 1
2600 MA DLEFT

NAME AND ADDRESS OF DEPOSITOR

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: Aspergillus niger DS2975	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: CBS 513.88

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

[ ] a scientific description

[X] a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 10-08-1988 (date of the original deposit)[1]

IV. INTERNATIONAL DEPOSITARY AUTHORITY

Name: Centraalbureau voor Schimmelcultures

Address: Oosterstraat 1
Postbus 273
3740 AG BAARN

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

Date: 7 December 1988
drs. G.B.A. van Reenen

[1] Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired; where a deposit made outside the Budapest Treaty after the acquisition of the status of international depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depositary authority.

• 91/EP 93 / 00356

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO

Gist-brocades
Wateringseweg 1
Postbus 1
2600 MA DELFT

NAME AND ADDRESS OF THE PARTY TO WHOM THE VIABILITY STATEMENT IS ISSUED

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the following page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Gist-brocades N.V. Address: Wateringseweg 1 Postbus 1 2600 MA DELFT	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: CBS 513.88 Date of the deposit or of the transfer[1]: 10 August 1988

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 31 August 1988[2]. On that date, the said microorganism was

[3] [X] viable

[3] [ ] no longer viable

[1] Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

[2] In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

[3] Mark with a cross the applicable box.

EPT/EP 93 / 00356

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED [4]

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: Centraalbureau voor Schimmelcultures

Address: Oosterstraat 1
Postbus 1
3740 AG BAARN

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

Date: 7 december 1988
drs. G.B.A. van Reenen

[4] Fill in if the information has been requested and if the results of the test were negative.

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 93/00356

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N9/96; A23K1/165

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; A23K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
Y	US,A,3 297 548 (JAMES H. WARE ET AL.) 10 January 1967 cited in the application see the whole document ---	1,5,9,11
Y	EP,A,0 420 358 (GIST-BROCADES N.V.) 3 April 1991 cited in the application see page 10, line 58; claims 28-31 ---	1,5,9,11
A	-/--	3,5

° Special categories of cited documents : [10]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
26 MAY 1993	11. 06. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	DEKEIREL M.J.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	BIOCATALYSIS vol. 1, 1988, GB pages 187 - 196 C. J. GRAY 'Additives and enzyme stability' cited in the application see page 189; table 1 see page 190, last paragraph - page 192, paragraph 2 ---	3-5
A	JOURNAL OF THE SCIENCE OF FOOD AND AGRICULTURE. vol. 54, no. 3, 1991, BARKING GB pages 355 - 365 V C NAIR ET AL. 'Production of phytase by Aspergillus ficuum and reduction of phytic acid content in canola meal' see page 362, paragraph 3 -paragraph 4; table 2 ---	1
A	EP,A,0 074 237 (JOHN & E STURGE LIMITED) 16 August 1983 see claims 1-7 -----	1

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

EP 9300356
SA 70461

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-3297548		DE-B- 1300488	
		GB-A- 1064304	
		NL-A- 6509747	31-01-66
EP-A-0420358	03-04-91	AU-A- 6501190	28-04-91
		CN-A- 1051058	01-05-91
		JP-T- 4506007	22-10-92
		WO-A- 9105053	18-04-91
EP-A-0074237	16-03-83	US-A- 4464469	07-08-84

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